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Biological and chemical diversity of cytotoxin-producing symbiotic marine fungi in intertidal zone of Dalian

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In this study, the biological and chemical diversity of 8 symbiotic marine fungal strains, with strong cytotoxicity against brine shrimp larvae, were investigated by nucleotide sequencing, morphology and cluster analysis of HPTLC fingerprint. These strains were identified by ITS rDNA sequencing, phylogenic analysis, and morphology to be *Hypocrea lixii*, *Chaetomium globosum*, *Aspergillus fumigatus*, *Asp. clavatus* and *Alternaria* sp. Their differences in secondary metabolites were shown by cluster analysis of digitalized colors of HPTLC spots, a newly developed method, which produced a similar dendrogram with that of ITS cluster analysis. Furthermore, this method can fully display intraspecific differences and even the remarkable difference in *Aspergillus* strains which goes beyond the boundary between genera. Their biological-chemical diversity may be the basis of their potent cytotoxicity and implies their potential in producing diversified antitumor or pesticidal constituents.

symbiotic marine fungi, cytotoxicity, diversity, rDNA sequencing, morphology, fingerprint

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Symbiotic fungi live on the surface or in the inner tissue of their hosts. Some terrestrial symbiotic fungi have been found to produce toxins or anti-feedants to protect their hosts from predators and grazers [1]. Some of these compounds can be used as antitumor or pesticidal agents. Symbiotic marine fungi have been isolated from seaweeds, sponges, corals, mangroves and sea grasses, also showing taxonomicall diversity and producing numerous active compounds [2].

The intertidal coastline of Dalian possesses diversified natural and artificial habitats and also high biodiversity of marine plants, invertebrates, and microorganisms. In our screening for useful cytotoxins from local symbiotic marine fungi using brine shrimp lethality test, a widely used bifunctional preliminary screening model to discover antitumor drugs and pesticides from the sea [3–5], eight strains with potent activities were discovered. Herein, we report the study on the biological and chemical diversity of these bioactive strains by ITS rDNA sequence analysis, morphology, and metabolite fingerprinting using a new cluster method.

1 Materials and methods

1.1 Bioactive strains under investigation

The eight symbiotic fungal strains, both epiphytes and endophytes, were isolated from the marine flora and fauna samples using the method previously reported [6]. The samples were collected from the intertidal zone of Fujiazhuang beach (121°36′13.82″E, 38°48′36.66″N) in Dalian City, China, in October of 2008 and May of 2009. These strains were statically fermented for 30 d in 200 mL of PSB (potato sucrose broth) containing 2% natural sea salt at 28°C. Mycelia were extracted with methanol and fermentation broth was extracted by ethyl acetate. The two extracts were combined to obtain crude organic extract,

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which was finally dissolved in 3 mL of methanol after rotary evaporation [6]. A total of 51 strains were screened for their remarkable cytotoxicity against brine shrimp larvae in the following bioassay.

The brine shrimp (*Artemia parthenogenetica*) larvae were hatched and collected using the method similar to that by Micheal et al. [7] and Lu et al. [8]. Fungal extracts and controls were respectively added into the microplates, and then dried in vacuum oven at room temperature. Afterwards, 200 μL of instar II-III *A. parthenogenetica* nauplii suspension containing 20–30 vivid larvae was added into each well. Then the microplates were incubated under fluorescent lamp in the incubator at 28°C for 24 h without cover. Pure water and methanol were respectively used as blank controls; taxol, adriamycin, and trichlorphon at final concentration of 5 μg/mL were used as positive controls. The results were observed and counted under a binocular dissecting microscope. The corrected average lethality rate of each sample was calculated according to Abbott Formula [9]. The bioassay consisted of 4 rounds of screenings with serially reduced dosage of extract and incubation time to select the strongest cytotoxin-producing fungal strains. For the four screenings, they were 40 μL/well for 24 h, 5 μL/well for 24 h, 1 μL/well for 24 h, and 1 μL/well for 4 h, respectively.

The information about the origin, crude extract content, and bioactivity of the eight strains is listed in Table 1.

1.2 Molecular taxonomy

DNA extraction of the strongest active fungal strains was performed using the Plant Genomic DNA Kit DP305 (Tiangen) according to the manufacturer's protocol. PCR was then performed using TaKaRa Ex *Taq* polymerase (TaKaRa) and the fungal universal primer pair ITS1 and ITS4, in a Takara PCR Thermal cycler Dice TP600 with the method of White et al. [10]. Then the PCR product mixture was analyzed by DNA electrophoresis on agarose gel, purified using TaKaRa DV805A Agarose Gel DNA Purification Kit, and sequenced by an ABI PRISMTM 3730XL DNA sequencer (TaKaRa) with primer ITS1. The sequence data had been submitted to and deposited at GenBank with the accession numbers shown in Table 2.

The ITS1-5.8 S-ITS2 (internal transcribed spacer, ITS) rDNA sequences were used to search the GenBank database with the BlastN 2.2.19+ algorithm for the closest matches in the ITS1-5.8 S-ITS2 rDNA sequences of known species. Sequences were aligned with representative fungal ITS1-5.8 S-ITS2 rDNA sequences using Clustal X (version 1.81); a neighbour-joining phylogenetic tree was constructed using the MEGA 4.0 [11,12].

1.3 Morphological characterization

Morphological characterization of the fungal isolates was carried out according to standard taxonomic key including colony diameter, texture, color, and the dimensions as well as the morphology of hyphae and conidia [13].

1.4 Cluster analysis of HPTLC fingerprint

For the strongest active strains, 1 μL of each extract was applied on high-performance thin-layer chromatographic plates (HPTLC silica gel 60 F254, Merck, Darmstadt, Germany) with a capillary. Then the HPTLC plate was developed twice by a mixture of $CHCl_3$/MeOH (20∶1, v/v). After air drying, the spots of the fungal metabolites on the plate were photographed under UV lamp at 254 and 365 nm, and then scanned after coloration by H_2SO_4-anisaldehyde agent at 105°C for 2 min.

To display the relationship and difference in secondary metabolism between these strains quantitatively, the metabolite fingerprints, i.e. the spots' colors on the HPTLC plates, were digitalized in the following section and analyzed by cluster method. Since TLC plate after chemical colorization gave more information, it was chosen for cluster analysis instead of UV TLC images. Firstly, the area between the

Table 1 The origin, crude extract yield and bioactivity of the 8 fungal strains[a)]

Strain number	Host	Type	Crude extract content in samples (mg mL^{-1})	Lethal rates of four rounds of brine shrimp lethality test (%)			
				1st	2nd	3rd	4th
DLEN2008003	*Gracilaria lemaneiformis*	endo-	43.2	100±0	100±0	100±0	18±12
6-N	*Chondrus ocellatus*	endo-	60.6	100±0	100±0	88±11	5±8
DLEN2008004	*Sargassum thunbergii*	endo-	109.1	100±0	100±0	96±3	100±0
13-F1	*S. kjellmanianum*	epi-	71.7	100±0	100±0	100±0	97±4
13-F2	*S. kjellmanianum*	epi-	44.0	100±0	100±0	85±9	95±5
11-N1	*Scytosiphon lomentarius*	endo-	69.6	100±0	100±0	100±0	99±2
DLEN2008006	*Reniera japonica*	endo-	57.1	100±0	100±0	100±0	0±0
DLEN2008010	*R. japonica*	endo-	67.9	100±0	100±0	100±0	27±10

a) The dosage of extracts and effect time for the 4 rounds of screenings were 40 μL×24 h, 5 μL×24 h, 1 μL×24 h and 1 μL×4 h, respectively. epi-: epiphytic; endo-: endophytic.

Table 2 The results of molecular and morphological identification of the active strains

Strain number	ITS length (bp)	Accession number	Closest reference strains	Identity (%)	Macroscopic and microscopic identification
DLEN2008003	581	HQ156781	*Hypocrea lixii* GQ328858	100	*Hypocrea* sp.
DLEN2008004	578	GU266272	*Hypocrea lixii* FJ442645	99	*Hypocrea* sp.
DLEN2008010	580	HQ149775	*Hypocrea lixii* FJ412025	99	*Hypocrea* sp.
6-N	541	GU244529	*Chaetomium globosum* FJ772001	99	*Chaetomium* sp.
13-F1	565	GU266273	*Aspergillus fumigatus* GQ169480	100	*Aspergillus* sp.
13-F2	554	HQ149772	*Aspergillus fumigatus* GU205082	99	*Aspergillus* sp.
DLEN2008006	567	GU266275	*Aspergillus clavatus* GU183170	99	*Aspergillus* sp.
11-N1	532	HQ149771	*Alternaria alternate GU797144*	100	*Alternaria* sp.
			Alternaria longipes AB470902	100	
			Alternaria brassicae FJ869872	100	
			Alternaria mali AY154683	100	
			Alternaria tenuissima AB369499	100	

baseline and the terminal line was equally divided into forty zones by horizontal lines. Secondly, a copy of the HPTLC plate image was opened in ACDSee software (version 3.1), by which each zone of each strain was cut out and saved as a new picture file. Then the new files were opened in Adobe Photoshop software and the eyedropper tool was used to select the main and representative color of the zones carefully. The hexadecimal code for the selected color can be read from the color picker. These codes are of optical implication. For white color, the number is the largest value FFFFFF; while for black color, the number is the smallest value 000000. For example, for zones 16–18 (*Rf* 0.374–0.442) of strain 13-F1, the color code for the pink spot was B83152. These hexadecimal codes can be transformed into decimal codes. For B83152, it was 12071250. Finally, the data, i.e., the decimal codes of zones 1–40 of each strain, were studied by SPSS software's (Version 13.0) Q-type hierarchical clustering method to compute the squred Euclidean distance and infer the dendrogram with its default settings.

2 Results

In the bioassay, strains 13-F1, 13-F2, DLEN2008006, 11-N1, DLEN2008003, DLEN2008004, DLEN2008010, and 6-N all showed lethality rates (LRs) of 100% in the first two rounds of screening and LRs over 85% in the third round (dosage: 1 μL/well, equivalent to 3.2–8.2 μg/mL for different samples; time: 24 h), which were comparable to or even stronger than the positive controls (86.9% for taxol, 37.4% for adriamycin, and 88.7% for trichlorphon at 5 μg/mL in 24 h) and much stronger than the remaining 43 strains (data not shown). Four of them showed extremely strong and acute toxicity against brine shrimp larvae with LRs of 95%–100% in the last round (Table 1). These facts indicated that these strains may be able to produce some powerful and interesting cytotoxins which would be useful in new drug development. Since the constituents of bioactive products are closely related with systematics of microorganisms, these strains were further investigated with respect to their taxonomy by nucleotide sequence analysis, morphology, and secondary metabolite fingerprints to understand their metabolic potential and diversity as cytotoxin producers.

2.1 Molecular and morphological taxonomy

First, the eight strongest strains were primarily characterized to be the members of different genera: *Aspergillus*, *Alternaria*, *Hypocrea*, and *Chaetomium*, respectively, according to their morphological characteristics of colonies, hyphae, and spores. Some of these features are easy to recognize, such as the actinomorphic conidiophore for *Aspergillus*, the dark septate clavate conidia for *Alternaria*, the grey-green colonies with rapid horizontal growth for *Hypocrea*, and the hairly perithecia for *Chaetomium*. They were further identified by molecular analysis on their ITS1-5.8S-ITS2 ribosomal DNA sequences with the length of 532–581 bp (Table 2).

The BLAST alignment of their ITS1-5.8S-ITS2 (simplified as ITS) rDNA sequences with the data stored in GenBank showed that strains DLEN2008003, DLEN2008004 and DLEN2008010 were 99%–100% similar to the reference strains of the species *Hypocrea lixii*, 6-N 99% similar to *Chaetomium globosum*, 13-F1 and13-F2 99%–100% similar to *Aspergillus fumigatus*, and DLEN2008006 99% similar to *Asp. clavatus*, respectively. In a phylogenic tree constructed by N-J algorithm (Figure 1), these strains were all placed on the corresponding clades with the reference species from GenBank (degree of confidence: 93%–100%), while branched from other related species of the same genera

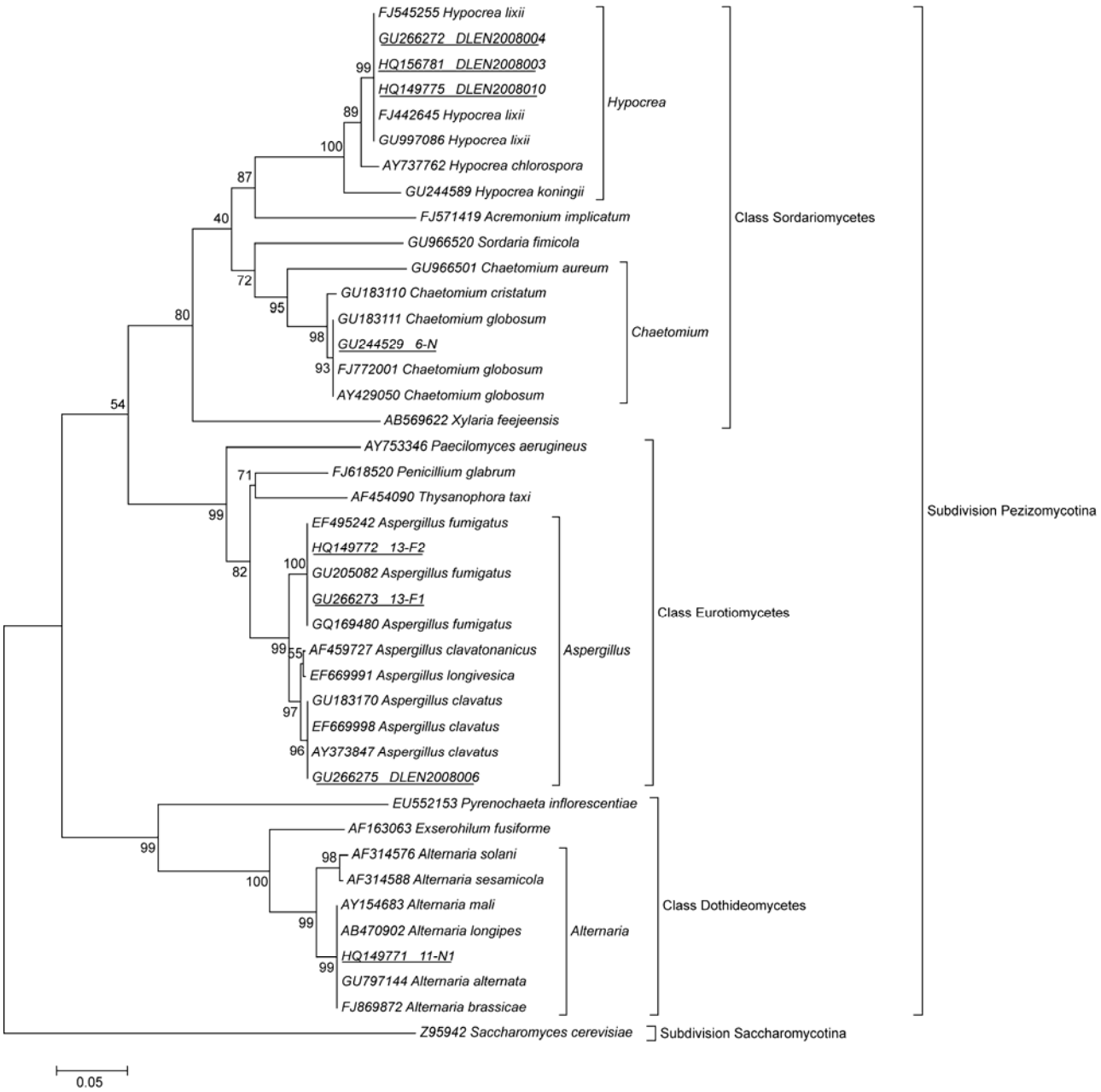

Figure 1 Neighbour-Jointing tree of ITS1-5.8S-ITS2 rDNA sequences of the top eight isolates (underlined), compared with sequences obtained from GenBank. Bootstrap values (expressed as percentages of 1000 replicates) are given at the nodes. The scale on the bottom indicates the number of substitutions per nucleotide. The Genbank accession numbers are listed before each isolate or reference strains.

(supported by 1000 bootstrap replicates).

As for strain 11-N1, the BLASTN revealed that it was highly similar to several small-spored species of genus *Alternaria* in GenBank with ITS rDNA's identities of 100%, including *Alt. alternata*, *Alt. brassicae*, *Alt. longipes* and *Alt. mali* and so on. In the phylogenic N-J tree (Figure 1), the 11-N1 isolate was also placed on the same clade with the above species (degree of confidence: 99%), while clearly branching from the large-spored group of this genus, e.g., *Alt. solani* and *Alt. sesamicola*. However, using only ITS sequence analysis, it was difficult to discriminate the high affinity species in small-spored group of genus *Alternaria* [14]. More professional morphological identification is needed to determine it to species level.

2.2 Analysis of secondary metabolites

HPTLC analysis revealed that these strongest active strains produced highly diverse metabolites as reflected by the spots under UV light or after chemical coloration (Figure 2). Furthermore, their profiles of products were quite different on genus and species levels and even within the same species

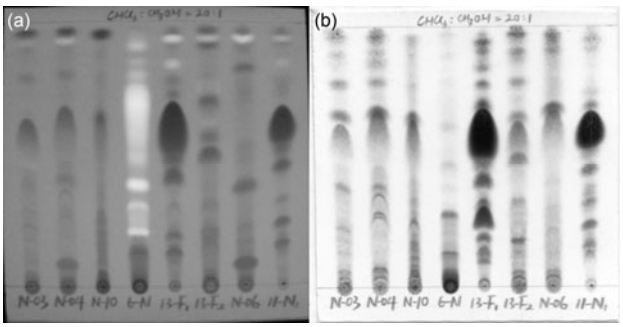

Figure 2 HPTLC analysis of the eight strongest strains (developed using Chloroform: Methanol=20∶1 (v/v) twice). (a) Under UV light at 254 and 365 nm; (b) coloration by H_2SO_4-anisaldehyde agent (the serial number of some strain were abbreviated, e.g. DLEN2008003 was simplified as N-03).

as shown by direct observation as well as by cluster analysis of fingerprint.

In the dendrogram of secondary metabolites (Figure 3), strains of genera *Hypocrea*, *Chaotomium* and *Alternaria* showed clear branches from each other; the relationship between them was consistent with the genetic phylogenic tree (Figure 1). Such relationship was also intuitively reflected by the disparate spots in the metabolites of 6-N (*Chaetomium globosum*) and 11-N1 (*Alternaria* sp.) in comparison with those of *Hypocrea lixii* strains DLEN2008003, DLEN2008004, and DLEN2008010. 6-N produced rich yellow compounds emitting white fluorescence under UV light, which became light orangish after coloration by H_2SO_4-anisaldehyde agent. 11-N1 produced rich colorful spots, e.g. vivid violet (*Rf* 0.58), pink (0.42) and purple (0.18) spots.

However, the three *Aspergillus* strains (*Asp. fumigatus* 13-F1 and 13-F2 and *Asp. clavatus* DLEN2008006) appeared not to be taxonomically restricted and were distributed among different genera as shown by their close relationship with *Alternaria* strain 11-N1 and *Hypocrea lixii* strains, respectively. Even though the strains 13-F1 and 13-F2 belong to the same species and were also isolated from the same host *Sargassum kjellmanianum*, the dendrogram showed a remote relationship between them; their TLC image also displayed quite different constituents in

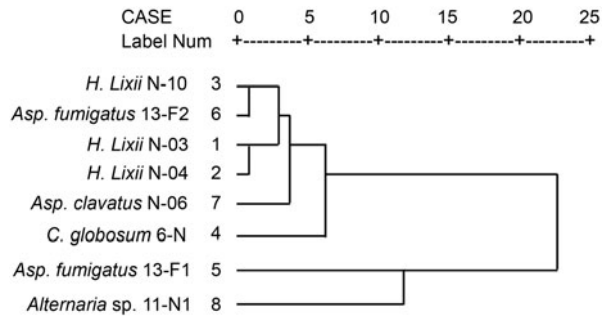

Figure 3 Cluster analysis of HPTLC fingerprint of the eight strongest strains by SPSS (the serial numbers of some strain were abbreviated, as shown in Figure 2).

high, middle, and low polar sections. This phenomenon for members of genus *Aspergillus* might be due to the variation on strain level and their powerful potential in the production of diverse secondary metabolites [2].

The secondary metabolite variation on strain level was also observed in *H. lixii* strains DLEN2008003, DLEN2008004, and DLEN2008010 from three different hosts. They were divided into two clades in dendrogram and their high polar constituents at *Rf* value of 0–0.42 were quite different. These variations on strain level will be useful in discovering diverse bioactive metabolites.

For strain 6-N, large-scale fermentation, extraction, chromatographic fraction, and activity tracing were preliminarily investigated in this study. The spots emitting white fluorescence at R_f of 0.46–0.77 and 0.18–0.41 as well as strong polar non-fluorescent constituents with R_f of 0 were found to be the main representative active components, which revealed the structural diversity of potential active compounds. Further purification and structure elucidation are underway.

To sum up, the analysis of secondary metabolism of these strongest active strains showed that they are highly chemically diverse and have the potential to produce active cytotoxins.

3 Discussion

3.1 Relationship between systematics and biological potential

As indicated by taxonomy, the symbiotic fungal strains with the strongest cytotoxicity primarily come from genera *Hypocrea* and *Aspergillus* and secondarily from *Chaetomium* and *Alternaria*. On class level, they belong to three different classes: Sodariomycetes, Eurotiomycetes and Dothideomycetes in the subdivision Pezizomycotina.

According to the reviews of Saleem et al. [15] and Blunt et al. [16–19], there are totally 80 marine fungal strains from 32 species as well as 4 unidentified strains that were reported to produce cytotoxic compounds in 2000–2009 worldwide. Among the former group, 79 strains in 31 species come from the subdivision Pezizomycotina of Ascomycota and only one strain in *Rhizopus* is from basal fungi occupying relatively lower position in the life tree of fungi. So far there is no report on Basidiomycota. In the cytotoxin-producing Pezizomycotina, 39 strains are reported from 4 species in Eurotiomycetes, 33 strains from 20 species in Sodariomycetes, and 7 strains from 7 species in Dothideomycetes. It seems that marine *Aspergillus* (20 strains reported) and *Penicillium* (15 strains reported) in Eurotiomycetes have the greatest potential in cytotoxin production. The members of the group with the second largest potential mostly come from Sodariomycetes, containing *Trichoderma* (4 strains), *Chaetomium* (3 strains), *Gliocladium* (4 strains), *Gymnascella* (3 strains), etc. It is interesting to note that half

of all the cytotoxin-producing *Sodariomycetes* species were from order *Hypocreales*. The relationship between systematics and bioactivity or cytotoxic compounds among the relevant genera/species is investigated in detail as follows.

Genus *Aspergillus* is known for its powerful secondary metabolic potential. On marine *Aspergillus* alone, there have been a large number of documents about producer strains of antitumor compounds with highly diverse structural types such as polyketides, peptides, alkaloids, terpenoids and lipids [20–24]. The reported producers include *Asp. glaucus*, *Aspergillus* sp. CNC-120, *Asp. fumigatus*, *Asp. ustus*, *Asp. terreus* and others from different hosts or sediments. Also reported is a marine algous *Asp. oryzae* producing indoloditerpenes against brine shrimp larvae [25]. Based on these reports, it may be easy to understand the highly heterogeneous HPTLC fingerprints and bioactivity of our three *Aspergillus* strains.

As we already know, terrestrial *Hypocrea* (anamorph *Trichoderma*) species are important biocontrol organisms against plant pathogens. Also, some marine *Hypocrea* strains from hosts, seawater or sediments are reported to possess larvicidal or antitumor activities, including *T. koningii* against blowfly larvae and strains which inhibit different cancer cell lines, e.g., a *Trichoderma* sp., *T. reesei*, *T. longibrachiatum* and *T. virens* [26–30]. Their diverse cytotoxic products are reported to include α-aminoisobutyric acid, dipeptides, cyclotetrapeptide, peptides, peptaibols, cyclopentenone and polyketide derivatives. Maybe some novel or known compounds in these or some other families can form the substantial basis for bioactivity of our cytotoxic *Hypocrea* strains.

Chaetomium globosum has been isolated from marine red alga *Polysiphonia urceolata*, green alga *Ulva pertusa* and fish *Mugil cephalus*. It is interesting that the three different strains also produced quite different families of antitumor compounds, i.e., benzaldehyde derivatives, cytochalasans and azaphilones [31–34].

A marine *Alternaria tenuis* from alga is also found to produce antitumor isocoumarin [35]. Besides, quite a few terrestrial *Alternaria* strains also give relevant reports. For example, tenuazonic acid and its derivative produced by *Alt. tenius* and plant endophytic *Alternaria* No. 28 showed pesticidal effects in field experiments and significant cytotoxic activity in brine shrimp bioassay, respectively [36,37]. Also reported were *Alt. porri* producing alterporriol F and plant endophytic *Alternaria* sp. producing 5 compounds against cancer cells [38,39].

As revealed by these documentary surveys, not only the cytotoxic activity but also the diverse active compounds from these taxa are closely related with systematics, which implies the great potential in producing diverse cytotoxins of genera *Hypocrea*, *Aspergillus*, *Alternaria* and *Chaetomium.* There are also reports on larvicidal activity (against brine shrimp) and related compounds. Our HPTLC analysis clearly indicated that the eight larvicidal strains can produce highly diverse secondary metabolites. Furthermore, the preliminary tracing of strain 6-N′ cytotoxins also showed the activity of different components with diverse structure-related properties. Therefore, these strains may be of importance in the search for novel cytotoxins to develop new antitumor agents and pesticides.

3.2 Cluster analysis of rDNA sequence and metabolite fingerprint in fungal taxonomy

For the 8 active strains, their ITS rDNA sequences and secondary metabolic fingerprints were both analyzed by cluster method in this study. The result showed that the ITS1-5.8S-ITS2 rDNA sequences were quite conservative on species level and well consistent with morphological taxonomy. They can be used as a convenient tool by nonprofessional researchers in fungal identification. However, it cannot entirely replace classic morphological taxonomy when dealing with some species with highly similar or completely identical ITS1-5.8S-ITS2 rDNA sequence such as *Alternaria* sp. Considering that different secondary metabolites can be represented by different colors of spots on TLC plates with specific R_f values after chemical colorization, the cluster analysis of HPTLC fingerprints was also applied in present study. The results showed that this method can give similar relationship as by genetic analysis and it can further exhibit the difference on intraspecific level. However, the strains in some species had extraordinarily strong secondary metabolism potential, e.g. *Aspergillus* spp., which may show clear branches or even cross the gaps between different genera. So this method may be taken as an assistant tool for intraspecific taxonomy and may also be suitable for intragenus taxonomy of highly similar species in some genera such as *Alternaria*.

Though cluster analyses of TLC fingerprint have been reported for plant and lichen taxonomy, previous reports mainly adopted principal component analysis (PCA) for some known or similar compounds from closely related plant samples, for which binary codes “0/1” were used to represent the absence or presence of specific metabolites [40–43]. Obviously, this method is not suitable for relationship analysis of entirely unknown and rich constituents of microorganisms, which are highly diverse taxonomically and chemically. The method in the present study resolved this question better by transforming the colors of different polar zones into standard hexadecimal codes to produce serial data for hierarchical cluster analysis. This method can be used as an assistant chemical taxonomical tool to analyze different microorganisms and macroorganisms. Although commercial scanner and manual color selection were used in this study due to the limitation of experimental conditions, satisfactory dendrogram was still obtained. If this method can be further improved and performed by automatic thin-layer scanner, it has the potential to be widely applied in chemical taxonomy.

4 Conclusions

In the selection of potential microbial strains for bioactive lead compounds' discovery and production, systematics by molecular genetic, morphological and chemtaxonomical methods can be a useful and rational guide. Based on ITS rDNA sequence, morphology, cluster analysis of HPTLC fingerprints, literature survey as well as preliminary toxins tracing, eight larvicidal symbiotic fungal strains were discovered to be diverse species/strains both biologically and chemically, with good prospect in cytotoxin production for medicinal or agrochemical purposes. Besides, the newly developed method of cluster analysis of HPTLC fingerprints by spot color digitalization and SPSS was able to obtain similar dendrogram with genetic phylogenic tree in the relationship analysis of fungal strains and show more detailed difference in secondary metabolism. This method may find extensive applications in chemotaxonomy after some modifications.

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